

Tissue plasminogen activator, von Willebrand factor and risk of type 2 diabetes in older men

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Received for publication 9 August 2007 and accepted in revised form 20 January 2008.

ABSTRACT

Objective: To assess the relationship between putative markers of endothelial dysfunction (tissue plasminogen activator antigen, t-PA; and von Willebrand factor antigen, vWF) and development of type 2 diabetes; and the role of inflammation, adipokines, hepatic function and insulin resistance in modifying these relationships.

Research Design and Methods: Prospective study of 3562 non-diabetic men aged 60-79 followed up for an average 7 years during which there were 162 incident type 2 diabetes.

Results: Elevated t-PA (top third) was associated with a near three-fold increase in risk of diabetes compared to those in the bottom third after adjustment for lifestyle factors and waist circumference (relative risk, RR 2.98; 95%CI, 1.79-5.00; $p < 0.0001$ for trend); weaker but significant (marginal) associations were seen with vWF (RR 1.24; 95%CI, 0.83-1.85; $p = 0.05$ for trend). Both biomarkers of endothelial dysfunction correlated significantly with markers of inflammation (interleukin-6 (IL-6), C-reactive protein (CRP)), hepatic function (gamma-glutamyl transferase (GGT)), and insulin resistance; with t-PA showing stronger associations with adiposity, hepatic function and insulin resistance than vWF. T-PA was also significantly and inversely associated with adiponectin. Adjustment for IL-6, adiponectin and GGT attenuated the association of incident diabetes with vWF (RR 1.06; 95%CI, 0.71-1.60) but the relationship seen with t-PA remained significant [adjusted RR 2.19; 95%CI, 1.29-3.70). Subsequent adjustment for insulin attenuated the association further but t-PA was still associated with a significant increase in risk (adjusted RR 1.66; 95% CI 0.96-2.85); $p = 0.02$ for trend).

Conclusion: T-PA antigen, but not vWF antigen, is independently associated with risk of type 2 diabetes.

Endothelial dysfunction plays a pivotal role in the development of atherosclerosis [1] and may be one of the underlying causes (or “common soil”) of both coronary heart disease (CHD) and type 2 diabetes [2]. There is substantial evidence suggesting a potential role for endothelial dysfunction in insulin resistance, although the relationship is almost certainly bi-directional [3]. A number of prospective studies have shown circulating biomarkers of endothelial dysfunction such as E-selectin, intercellular adhesion molecule 1 (ICAM-1), von Willebrand factor (vWF), and in particular plasminogen activator inhibitor type 1 (PAI-1) to predict incident diabetes [4-9] although the findings have not always been consistent. Some studies have shown the above associations to be independent of inflammation and insulin resistance [6,7]. Adipokines, in particular low adiponectin, and hepatic function as measured by the hepatic enzymes alanine aminotransferase and gamma-glutamyl transferase (GGT) have been associated with risk of subsequent type 2 diabetes [10-13]. Adipokines and liver dysfunction have also been linked to PAI-1 and markers of endothelial dysfunction [14-16]. Few population studies have addressed the associations between liver dysfunction and markers of endothelial dysfunction. Whether the association between markers of endothelial dysfunction and diabetes is independent of adipokines and hepatic function has not been studied, but is important to do so to assess possible residual confounding.

Tissue plasminogen activator (t-PA) is released from vascular endothelium and hence circulating levels of t-PA antigen may be a marker of endothelial dysfunction [17]. However higher plasma t-PA antigen represents largely inactive circulating t-PA-PAI-1 complexes which in turn, may reflect both endothelial disturbance (t-PA and PAI-1 release) and hepatic PAI-1 release [17]. Elevated t-PA antigen is considered an integral feature of the insulin resistance

syndrome and is also related to the inflammatory response [18]. The results of one population study suggest that t-PA is predictive of future diabetes independent from the metabolic syndrome [19], although this study was limited in the small number of incident cases and the findings were not statistically significant. This question is worthy of study since t-PA is more stable than PAI-1 and also appears to be more strongly linked to incident CHD events in population cohorts [17]. We have therefore examined the relationships between t-PA as well as vWF (two circulating markers of endothelial dysfunction) and the risk of incident type 2 diabetes; and have assessed whether these associations are independent of adiponectin, hepatic function as measured by ALT and GGT levels, inflammation as measured by levels of C-reactive protein (CRP) and interleukin-6 (IL-6), and insulin resistance.

SUBJECTS AND METHODS

The British Regional Heart Study is a prospective study of cardiovascular disease involving 7735 men aged 40-59 years selected from the age-sex registers of one general practice in each of 24 British towns, who were screened between 1978 and 1980 [20]. In 1998-2000, all surviving men, now aged 60-79 years, were invited for a 20th year follow-up examination. All relevant local research ethics committees provided ethical approval. All men provided informed written consent to the investigation, which was carried out in accordance with the Declaration of Helsinki. They completed a questionnaire (Q20) which included questions on their medical history, lifestyle behaviour and family history of diabetes. The men were requested to fast for a minimum of 6 hours, during which time they were instructed to drink only water and then to attend for measurement at a specified time between 0800 and 1800h. All men were asked to provide a blood sample, collected using the Sarstedt Monovette

system. 4252 men (77% of survivors) attended for examination.

Cardiovascular risk factors. Details of measurement and classification methods for smoking status, physical activity, body mass index, social class, alcohol intake, blood pressure and blood lipids in this cohort have been described [20-22]. Anthropometric measurements including body weight, height and waist circumference (WC) were carried out. Subjects were measured in light clothing without shoes in the standing position. Body mass index (BMI; weight/height² in kg/m²) was calculated for each man. From the combined information at initial screening and follow-up questionnaires in 1996 (Q96) and at rescreening (Q20), the men were classified into 5 smoking groups: (i) those who had never smoked, (ii) ex-smokers since screening, (iii) smokers at baseline who gave up between screening and Q96, (iv) smokers at baseline and at Q96 who gave up after 1996, and (v) current cigarette smokers at Q20. The longest held occupation of each man was recorded at screening and the men were grouped into one of six social classes: I, II, III non-manual (non-manual groups), III manual, IV and V (manual groups). Those whose longest occupation was in the Armed Forces formed a separate group. Heavy drinking is defined as >5 drinks/day. On the basis of a physical activity score [21] the men were classified into 4 groups: inactive, light, moderate, moderately vigorous/vigorous. Plasma glucose was measured by a glucose oxidase method using a Falcor 600 automated analyser. Serum insulin was measured using an ELISA assay which does not cross-react with proinsulin. Triglycerides, blood glucose and insulin concentrations were adjusted for the effects of fasting duration and time of day [22]. Insulin resistance was estimated according to the homeostasis model assessment (HOMA - IR - the product of fasting glucose (mmol/L) and insulin (units/mL) divided by the constant 22.5) [23]. Plasma levels of t-PA antigen were measured with an enzyme-linked immunosorbent assay (Biopool AB,

Umea, Sweden) as was von Willebrand factor (vWF) antigen (DAKO, High Wycombe, UK) [21]. C-reactive protein was assayed by ultra sensitive nephelometry (Dade Behring, Milton Keynes, UK). Plasma adiponectin concentrations were determined using ELISA (R&D systems, UK) [10]. Interleukin-6 was assayed using a high-sensitivity ELISA (R & D Systems, Oxford, UK[10]). Hepatic enzymes including GGT and alanine transaminase (ALT) were measured using a Hitachi 747 automated analyser.

Study subjects. Endothelial marker measurements (t-PA or vWF) were available in 4083 men at Q20. Men with a doctor diagnosis of diabetes and men diagnosed with diabetes in year of re-examination and those with a fasting glucose of > 7 mmol per litre (WHO criteria) were considered to have prevalent diabetes and were excluded (n=484). We further excluded men with missing data on IL6 (n=37). Thus analysis is based on 3562 men.

Follow-up. All men have been followed up for all cause mortality, cardiovascular morbidity and development of type 2 diabetes from initial examination to July 2006 [24] and follow-up has been achieved for 99% of the cohort. This analysis is based on follow-up from re-screening in 1998-2000, a mean follow-up period of 7 years (6-8 years). Information on deaths was collected through the established "tagging" procedures provided by the National Health Service registers. Evidence regarding diabetes were obtained by reports from general practitioners, by biennial reviews of the patients' notes (including hospital and clinic correspondence) through to the end of the study period. Cases are based on self-reported diagnoses confirmed by primary care records; an approach which has been validated in the present study [25].

Statistical Methods. The men were divided by tertiles of the inflammatory markers. Cox's proportional hazards model was used to assess the multivariate-adjusted relative risk for each third compared with the reference group (lowest third). In the

adjustment, smoking (never, long term ex-smokers (>15 years), recent ex-smokers and current smokers), social class (7 groups), physical activity (4 groups), alcohol intake (5 groups), parental history of diabetes (yes/no), pre-existing CHD (yes/no) and stroke (yes/no), use of statins (yes/no) were fitted as categorical variables. WC, HOMA-IR, GGT, adiponectin, IL-6, CRP were fitted as continuous variables.

RESULTS

During the mean follow-up period of 7 years (range 5-7 years) there were 162 incident cases of diabetes in the 3562 non-diabetic men. Table 1 shows the baseline characteristics in the men who developed diabetes and in men who remained free of diabetes. Men who developed diabetes had higher BMI and WC than those who did not. They were also more likely to be physically inactive, to have a parental history of diabetes, higher prevalence of CHD, higher levels of metabolic risk factors and to have significantly higher levels of markers of inflammation (CRP, IL-6), endothelial dysfunction markers (vWF and t-PA), higher levels of hepatic enzymes (GGT, ALT) and lower adiponectin levels than men who did not develop diabetes.

Table 2 shows the correlations between endothelial dysfunction markers and their correlations with metabolic risk factors, inflammation, adiponectin and hepatic enzymes. T-PA antigen was strongly correlated with central adiposity (WC) and was significantly associated with all components of the metabolic syndrome, insulin resistance, adiponectin and hepatic enzymes. These associations persisted even after adjustment for WC (Table 2). VWF showed weaker but significant associations with WC, hepatic enzymes and insulin resistance. Less consistent associations were seen for the metabolic risk factors. All showed similar significant associations with inflammatory markers (CRP, IL-6).

Table 3 shows the incidence rates for diabetes and the relative risk of diabetes by tertiles of the biomarkers of endothelial

dysfunction with adjustment for demographic factors (age, social class, smoking, physical activity, alcohol intake, parental history of diabetes, pre-existing CHD and use of statins) and the effects of adjustment for WC, inflammation (IL-6), adiponectin, hepatic enzymes (GGT) and insulin resistance (HOMA-IR). IL-6 was used in the adjustment as IL6 and CRP are strongly correlated ($r=0.57$) and CRP showed no independent association with diabetes after adjustment for IL-6. Similarly GGT and ALT are strongly correlated ($r=0.43$) and GGT was a stronger predictor of diabetes than ALT [17] and showed stronger associations with the endothelial dysfunction markers.

Elevated t-PA was associated with over a twofold increase in risk of diabetes even after adjustment for demographic factors and WC (Model 2). WC and BMI are highly correlated ($r=0.86$). The findings were virtually unchanged if BMI was included in the adjustment instead of WC. A weaker association was seen between vWF and diabetes. To further assess whether the relationship between diabetes and markers of endothelial dysfunction was independent of inflammation and adipocytes and hepatic enzymes we adjusted further in turn for adiponectin, IL-6 and GGT. Simultaneous adjustment for these factors attenuated the associations but the increased risk of diabetes associated with elevated t-PA remained significant. No association was seen with vWF after adjustment.

Insulin resistance could mediate the association between t-PA and risk of diabetes. Adjustment for HOMA-IR in addition to IL-6 and adiponectin (Model 3) considerably attenuated the relationships for t-PA, but it remained significantly associated with increased risk of diabetes. When GGT and HOMA-IR were both included in the adjustment (Model 3 +GGT+HOMA-IR) elevated t-PA was still associated with a 60% increase in risk.

The increased risk of diabetes associated with elevated t-PA was seen in both obese and centrally obese (WC >102 cm or BMI

>30 kg/m²) and non obese (centrally obese) men and in those with normal glucose levels (<6.1 mmol/l) and in those with impaired glucose levels (>6.1 mmol/l) (data not shown). No significant interaction was seen between obesity or glucose levels and t-PA and risk of diabetes (p=0.41 and p= 0.50 for obesity and high glucose, respectively).

DISCUSSION

In this large random sample of non-diabetic British men aged 60-79 years, we have confirmed the findings of several previous studies [4-9] that circulating markers of endothelial dysfunction are associated with incident diabetes. We have shown that t-PA antigen, but not vWF antigen, is independently associated with the development of diabetes in men. Our findings extend those of other studies on circulating endothelial markers and risk of type 2 diabetes by assessing a wider range of risk factors and adjusting for correlates reflective of perturbances in other relevant pathways including markers of inflammation, insulin resistance as well as adiponectin and hepatic enzymes, which have not previously been assessed or comprehensively adjusted for. The association between vWF and diabetes was largely dependent on inflammation. Adjustment for IL6 attenuated the association between vWF and risk of diabetes. By contrast, the association between t-PA antigen and diabetes was independent of markers of adiposity or inflammation, adiponectin and hepatic enzymes, despite being correlated with markers in each pathway. The increased risk associated with elevated t-PA partially reflected insulin resistance, but there still remained a 60% increase in risk even after adjustment for HOMA-IR. We considered vWF and t-PA to reflect endothelial dysfunction; however the modest correlation between these two biomarkers (r=0.21) and the differing associations seen between t-PA and vWF with insulin resistance and features of the metabolic syndrome support the view that these endothelial biomarkers are

regulated differently; therefore, one should be cautious in labelling such markers equally or purely under the endothelial function 'umbrella'.

VWF is produced mainly by vascular endothelial cells activated by proinflammatory cytokines [26]. Although vWF was shown to be predictive of diabetes independent of a marker of inflammation (CRP) and insulin in the Framingham Offspring Study [7], others have reported no association [8] or no independent association between vWF and diabetes [27]. Our results generated in a more homogenous population of predominantly white men suggest that the association between vWF and diabetes is explained by levels of the proinflammatory cytokine IL-6, which is known to be associated with incident diabetes [10], and which stimulates release of vWF from vascular endothelium [28]. Thus, any link between vWF and incident diabetes may be indirect rather than direct.

Circulating t-PA antigen reflects not only endothelial synthesis and release of t-PA, but also circulating complexes of t-PA with its major inhibitor, PAI-1, [17] which is synthesised by adipocytes, hepatocytes and endothelial cells and is also an acute-phase reactant. T-PA (and PAI-I) levels increase as part of the inflammatory response [29]; and in the present study t-PA correlated with several inflammatory markers including CRP and IL-6. Numerous studies have shown PAI-1 to be predictive of type 2 diabetes [4,6,7,8] and in some to be independent of adiposity, and markers of inflammation [6,7]. Similar relationships were seen for t-PA in this study, as one might expect given its strong correlation with PAI-I [26]. Inflammation and adiponectin appeared to have only modest attenuating effects on the relationship between t-PA and diabetes. In our previous report we have shown IL6 (positively) and adiponectin (inversely), to be predictive of diabetes [11]. Since t-PA levels correlated with IL-6 and adiponectin, t-PA may explain the positive relationship between IL6 and diabetes seen in this and other studies and

the significant inverse association seen between adiponectin and diabetes in obese men [11]. However, these relationships remained significant after adjusting for t-PA. Thus the association between t-PA, adipokines (IL6 and adiponectin) and diabetes appear to be independent of each other.

Insulin has been shown to stimulate expression of PAI-1 and t-PA [30]. T-PA was associated with insulin resistance and all components of the metabolic syndrome, and the relationship between t-PA and diabetes was to some extent explained by insulin resistance, but there remained a significant independent association after adjustment for HOMA-IR. Clearly, more direct measures of insulin resistance e.g. clamp, may have attenuated the relationship between t-PA and incident diabetes further. Indeed, we have previously reported strong associations between clamp measured insulin resistance (M/I) and t-PA antigen concentration in women with polycystic ovarian syndrome ($r = -0.59$; $P < 0.05$) and healthy controls ($r = -0.62$; $P < 0.05$) [31]. Thus t-PA antigen concentrations, partly by virtue of strong correlations to insulin resistance, may be useful as part of a multiple biomarker portfolio to predict future diabetes risk.

The association of t-PA and diabetes may also partially occur via common associations with liver disturbance. Indeed, metformin, which is thought to work principally at the liver, has been consistently shown to lower t-PA concentrations in different groups, and to do so in correlation to improvements in insulin resistance measures [32]. The association between t-PA and diabetes was attenuated further but not eliminated after inclusion of both insulin resistance and hepatic function in the adjustment. Prospective studies relating t-PA to diabetes are extremely limited. Our finding that t-PA predicted diabetes is consistent with a much smaller previous report of t-PA in the Northern Sweden MONICA Study [19], which also considered only a limited number of potential

confounders. Thus our work extends the available data on the link between t-PA and incident diabetes.

Our study is not without some limitations. Our study was carried out in an older predominantly white Caucasian male population and we cannot generalise our findings to women, younger men or other ethnic groups although, as noted above, t-PA has been shown to be strongly correlated with measured insulin resistance in women with polycystic ovarian syndrome and controls [31], suggesting that the t-PA diabetes relationship is likely to be seen in women as well. Diabetes incidence in this study relied on documented doctor diagnosed cases of diabetes, which would inevitably result in under-ascertainment of cases. It may be argued that those with elevated markers of endothelial dysfunction are more likely to be obese and to have had contact with their general practitioner and thus more likely to have had diagnostic tests for diabetes. However, among the prevalent diabetics at re-screening who had been excluded, t-PA and vWF were positively associated with both diagnosed diabetes (physician diagnosis) and undiagnosed diabetes (using fasting glucose concentration criteria of > 7.0 mmol/l). Thus the increased risk associated with elevated markers of endothelial dysfunction is unlikely to be due to bias in ascertainment. Finally we did not measure PAI-1 directly, but note that t-PA is the more stable marker [33].

Circulating levels of t-PA [17] and vWF [26] are associated with risk of coronary heart disease (CHD), and their elevations in persons with type 2 diabetes [34] might therefore promote their atherothrombotic complications. The present study, adds to the literature on the potential importance of t-PA in the development of diabetes. We suggest that further, detailed studies of endothelial markers (in particular t-PA) and risk of diabetes be performed, to clarify their value in prediction of diabetes, ideally in combination with other markers, and their possible roles in pathogenesis.

ACKNOWLEDGEMENTS

The British Regional Heart Study is a Research Group supported by the British Heart Foundation. The measurements and laboratory analyses reported here were supported by British Heart Foundation Project Grants PG97012 and PG97027. We thank Karen Craig, Estelle Poorhang, Paul Welsh and Fiona Key for technical support.

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TABLE 1.

Distribution of risk factors and inflammatory/haemostatic markers in 3562 non-diabetic subjects aged 60-79 years at re-examination according to diabetes status at follow-up.

	Developed diabetes		p-value difference
	No N=3400	Yes N=162	
Age, years	68.7 (5.6)	68.4 (5.4)	0.56
BMI, kg/m ²	26.6 (3.5)	29.7(4.1)	<0.0001
WC, cm	96.2 (9.9)	104.1 (10.4)	<0.0001
% current smokers	13.3	9.3	0.14
% inactive	32.4	45.2	0.0008
% manual	53.1	64.6	0.004
% heavy drinkers (>5drinks/day)	3.7	2.5	0.42
% parental history of diabetes	5.4	9.9	P<0.0001
% CHD	17.6	32.1	<0.0001
% use of statins	6.5	13.6	0.0005
% stroke	5.0	6.2	0.57
SBP, mm Hg	148.2 (23.9)	153.0 (22.7)	0.01
Triglyceride [#] , mmol/l	1.57 (1.12-2.13)	2.10 (1.54-2.81)	<0.0001
HDL, mmol/l	1.34 (0.34)	1.17 (0.29)	<0.0001
Glucose [#] , mmol/l	5.52 (5.21-5.89)	5.99 (5.59-6.52)	<0.0001
Log HOMA-IR	0.65 (0.58)	1.27 (0.57)	<0.0001
CRP [#] , mg/L	1.67 (0.80-3.30)	2.59 (1.27-4.42)	<0.0001
IL-6 [#] , pg/ml	2.41 (1.55-3.42)	2.92 (1.93-4.23)	<0.0001
Adiponectin [#] , µg/ml	7.11 (4.52-11.46)	4.96 (3.11-7.39)	<0.0001
GGT [#] , IU/L	27.4 (18-37)	37.0 (24-52.5)	<0.0001
ALT [#] , IU/L	15.2 (12-20)	19.5 (13.5-28.0)	<0.0001
VWF, iu/dL	137.8 (44.4)	146.4 (48.1)	0.02
t-PA, ng/mL	10.74 (4.21)	13.37 (4.79)	<0.0001

Data are means (SD), geometric means (interquartile range) for skewed variables

geometric mean

TABLE 2. Spearman correlation coefficients of endothelial markers with metabolic and anthropometric variables, inflammatory markers, liver enzymes and adiponectin.

	t-PA		t-PA		vWF		vWF	
	Unadjusted		Adjusted For WC		Unadjusted		Adjusted For WC	
	r	<i>p-value</i>	r	<i>p-value</i>	r	<i>p-value</i>	r	<i>p-value</i>
t-PA	1.00				0.21	<0.0001	0.20	<0.0001
VWF	0.21	<0.0001	0.20	<0.0001	1.00			
Metabolic factors								
WC	0.31	<0.0001			0.06	<0.001		
HDL-C	-0.20	<0.0001	-0.12	<0.0001	-0.07	<0.001	-0.04	<0.01
Triglycerides	0.34	<0.0001	0.26	<0.0001	0.03	<i>ns</i>	0.02	<i>ns</i>
SBP	0.09	<0.0001	0.07	<0.0001	-0.01	<i>ns</i>	-0.02	<i>ns</i>
Glucose	0.13	<0.0001	0.11	<0.0001	0.04	<i>ns</i>	0.03	<i>ns</i>
HOMA	0.35	<0.0001	0.25	<0.0001	0.11	<0.0001	0.10	<0.01
Inflammatory markers and adiponectin								
CRP	0.25	<0.0001	0.20	<0.0001	0.26	<0.0001	0.25	<0.0001
IL-6	0.20	<0.0001	0.16	<0.0001	0.25	<0.0001	0.24	<0.0001
Adiponectin	-0.15	<0.0001	-0.12	<0.0001	0.06	<0.001	0.07	<0.001
Liver enzymes								
ALT	0.17	<0.0001	0.11	<0.0001	0.005	<i>ns</i>	-0.005	<i>ns</i>
GGT	0.31	<0.0001	0.27	<0.0001	0.13	<0.0001	0.12	<0.0001

TABLE 3. Incidence rates and adjusted relative risk of type 2 diabetes according to thirds of inflammatory and endothelial markers.

		Thirds		trend
Endothelial markers				
t-PA, ng/mL	<8.7	8.7-	12.1	
Rate/1000 per-yrs	2.6	6.3	11.6	
Model 1	1.00	2.36 (1.40,3.99)	4.54 (2.76,7.47)	<0.0001
Model 2	1.00	1.89 (1.12,3.20)	2.98 (1.79,5.00)	<0.0001
Model 3	1.00	1.81 (1.07,3.07)	2.65 (1.58,4.45)	<0.0001
Model 3+GGT	1.00	1.65 (0.97,2.81)	2.19 (1.29,3.70)	0.0003
Model 3+HOMA-IR	1.00	1.35 (0.78,2.32)	1.92 (1.12,3.27)	0.001
Model 3 +GGT+HOMA-IR	1.00	1.25 (0.72,2.16)	1.66 (0.96,2.85)	0.02
VWF, iu/Dl	<115	115-	155-	
Rate/1000 per-yrs	5.8	6.1	8.4	
Model 1	1.00	1.04 (0.70,1.56)	1.39 (0.94,2.03)	0.007
Model 2	1.00	0.94 (0.62,1.41)	1.24 (0.83,1.85)	0.05
Model 3	1.00	0.91 (0.61,1.38)	1.18 (0.79,1.78)	0.11
Model 3+GGT	1.00	0.87 (0.57,1.31)	1.06 (0.71,1.60)	0.31
Model 3+HOMA-IR	1.00	0.87 (0.57,1.32)	1.10 (0.73,1.66)	0.27
Model 3 +GGT+HOMA-IR	1.00	0.84 (0.55,1.28)	1.01 (0.67,1.53)	0.49

Model 1 =adjusted for age, social class, smoking, alcohol intake, physical activity, parental history of diabetes, pre-existing CHD, stroke and use of statins.

Model 2 =Model 1+ waist circumference

Model 3=Model 2+adiponectin and IL6